

Metabolism and Activation Pathways of the
Environmental Mutagen 3-Nitrofluoranthene

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ENVIRONMENTAL MUTAGEN 3-NITROFLUORANTHENE

by

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16. ABSTRACT Nitro-substituted polycyclic aromatic hydrocarbons are widespread environmental contaminants that are mutagenic and potentially carcinogenic. 3-Nitrofluoranthene (3-NFA) has been found in diesel and ambient air particulate extracts; although less abundant than its isomer 1-nitropyrene (1-NP), its content in these extracts correlates well with their direct-acting mutagenic potency in the Ames assay. 3-NFA is more mutagenic towards Salmonella TA98 than 1-NP both without (2 500 His ⁺ rev/nmol vs. 350 rev/nmol) and with exogenous metabolic activation (250 vs 60 rev/nmol). 3-Amino-fluoranthene was less active than 3-NFA both with and without S9 (100 and 40 rev/nmol respectively); 3-acetamidofluoranthene was even less active with S9 (60 rev/nmol), and quite inactive without S9. In contrast, 1-acetamidopyrene (400 rev/nmol +S9) was more active than the parent 1-NP. The activity of 3-NFA was substantially reduced in the nitroreductase-deficient strains of TA98 (TA98NR and TA98/1,8-DNP ₆), indicating that the "classical" nitroreductase and the transacetylase enzymes are involved in activation of 3-NFA. The presence of S9 reduced the activity of 3-NFA in all assays except the forward mutation assay in strain TM677, where it was substantially enhanced. 3-NFA is readily metabolised by rodent hepatic subcellular fractions (at rates of the order of 25 nmoles/mg protein/hr). Hydroxylated NO ₂ PAH in an ambient air particulate extract have been tentatively identified as 3-NFA derivatives, and contribute significantly to the mutagenicity of this extract. Therefore as with 1-NP, oxidation may play a role in metabolic activation of 3-NFA.					
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METABOLISM AND ACTIVATION PATHWAYS OF THE ENVIRONMENTAL
MUTAGEN 3-NITROFLUORANTHENE.

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INTRODUCTION

The presence of nitrated polycyclic aromatic hydrocarbons (NO₂PAH) in photocopier toners (1), in diesel and woodsmoke combustion emissions (2,3,4), and in ambient air (4,5), has generated concern about possible adverse health effects arising from the widespread environmental occurrence of these compounds many of which are potent mutagens in the Ames Salmonella typhimurium plate incorporation assay (6). 1-Nitropyrene (1-NP), abundant in extracts of diesel particulate matter, has been extensively characterised as a representative NO₂PAH, with respect to mutagenicity in Salmonella (6,7) and mammalian (8) assay systems, metabolism both in vivo (9,10) and in vitro (10, 11,12,13,14), formation of adducts with DNA (13,14), and animal carcinogenicity (15,16). 3-Nitrofluoranthene (3-NFA) has been reported as a potent bacterial mutagen (17) and possibly an animal carcinogen (15), but little was known of its mechanisms of activation and to what extent nitroreductase enzymes (as is the case for other NO₂PAH [7]) were involved in generation of genotoxic intermediates. On account of the particular abundance of fluoranthene and its derivatives in ambient air particulate matter (5), we have undertaken to investigate further the mutagenicity, metabolism and activation pathways of 3-NFA.

MATERIALS AND METHODS

Chemicals

3-Nitro[3-¹⁴C]fluoranthene (¹⁴C-3-NFA) and unlabelled 3-NFA were synthesised by Midwest Research Institute, Kansas City, MO. 3-Aminofluoranthene (3-AFA) was purchased from Aldrich Chemical Co., Milwaukee, WI, and recrystallised from aq. EtOH until yellow flakes (m. pt. 107-108°C; lit. 113-115°C [18]) were obtained which were demonstrated by HPLC analysis to be

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over 99.5% pure (judged by monitoring UV absorbance at 254 nm). 3-Acetamidofluoranthene (3-AAFA) was prepared by acetylation of 3-AFA and recrystallised from aq. EtOH to yield orange-yellow needles, m. p. 243.5-245°C (lit. 244-245 C [18]); by HPLC analysis (254 nm) over 99.5 % of the product chromatographed as a single peak. MS and proton NMR spectra were consistent with the proposed structures. Other chemicals and solvents were bought from Sigma Chemical Co., St. Louis, MO, Burdick and Jackson Inc., Muskegon, MI and Fisher Scientific, Raleigh, NC.

Mutagenicity Assays

The *Salmonella typhimurium* plate incorporation assay was carried out as described by Ames et al. (19), with the following modifications: minimal histidine was added to the base agar rather than to the soft agar overlay, and the number of histidine-prototrophic revertant colonies was counted (Artek Colony Counter, Farmingdale, NY) after 72 rather than 48 h. *Salmonella* strain TA98 was obtained from Dr. B. Ames, University of California, Berkeley, CA, USA, and strains TA98NR and TA98/1,8-DNP₆ from Dr. H.S. Rosenkranz, Case Western Reserve University, Cleveland OH, USA. The S9 fraction (9000xg supernatant) used to provide metabolic activation was prepared from the livers of Aroclor 1254-treated male CD-1 rats (Charles River, Wilmington, MA, USA), stored at -70°C until used, and supplemented with NADPH-generating co-factor mix as described (19). The amount of S9 protein added (1.2 to 1.5 mg per plate) was determined by optimisation with benzo(a)pyrene and 2-aminoanthracene for each batch of S9 prepared. Each compound was assayed in triplicate, on three separate occasions, at doses ranging from 0.01 to 10 µg per plate, both with and without exogenous metabolic activation (S9). Compounds were freshly dissolved in DMSO before each assay. Specific mutagenicity was calculated from the linear portion of the dose-response curve.

The forward mutation assay to 8-azaguanine resistance was performed scaled down ten-fold from the method of Skopek et al. (20), as described by Goto et al. (21), in a bacterial strain cloned from a culture of *Salmonella typhimurium* TM677 supplied by Dr. W.G. Thilly, Massachusetts Institute of Technology, Cambridge, MA, and selected for maximum activity towards benzo(a)pyrene. 3-NFA dissolved in DMSO was incubated with bacteria (and S9 mix) for 1 h, then triplicate plates (for each dose) were poured for scoring of resistant mutants and of survivors.

Incubation Of 3-Nitro[3-¹⁴C]fluoranthene With S9

¹⁴C-NFA (or unlabelled 3-NFA; final concentration 50 µM)

dissolved in DMSO was added to Aroclor-treated rat liver S9 fraction (1 mg/ml in 0.1M HEPES buffer, pH 7.4; final vol. 80 ml) prepared as described (19). The reaction was started by addition of NADPH (1mM) and terminated (after 1 h at 37°C) by addition of 1 vol. of ice-cold ethyl acetate/acetone (2:1,v/v). The reaction products were extracted with a further 2 x 1 vol. of ethyl acetate/acetone. The pooled organic extracts were concentrated (to approx. 3 ml) by rotary evaporation, then taken to dryness under a stream of N₂ gas. The residue was dissolved in MeOH (1 ml) for further analysis. Recovery of ¹⁴C was monitored throughout by liquid scintillation counting.

Analysis and Quantitation of 3-Nitrofluoranthene Metabolites

The profile of ¹⁴C organic-extractable metabolites was determined by HPLC with a gradient from 70 to 100 % MeOH in H₂O (rising at 2 %/min) and liquid scintillation counting of 30-sec fractions of the eluate. The H₂O-soluble metabolites were similarly analysed over a gradient from 0 to 100% MeOH in H₂O (2% /min); fractions were collected and counted every min. The remainder of the ¹⁴C-extractable fraction and of parallel incubations conducted with unlabelled 3-NFA were chromatographed over a gradient from 50 % MeOH in H₂O to 100 % MeOH; fractions were collected according to the UV (254 nm) absorbance of the eluate.

Instrumentation

Proton NMR spectra were recorded in acetone-d₆ on a Bruker WM-250 instrument. Mass spectra were obtained in the direct probe insertion EI mode (70 eV) on a VG7070H (Vacuum Generators, Altrincham, Cheshire) mass spectrometer and by HRGC/NCI-MS on a Finnegan gas chromatograph/EI-CI mass spectrometer (Finnegan Instrument Co., Sunnyvale, CA). Melting points were determined on a Hoover capillary melting point apparatus (A.H.Thomas Co., Philadelphia, PA) and are uncorrected throughout. UV-vis spectra were recorded on a Cary 2200 spectrophotometer (Varian Techtron Pty Ltd, Mulgrave, Australia), and fluorescence spectra on an in-line Perkin-Elmer MPF-2A fluorometer (Perkin-Elmer, Norwalk, CT). Analytical HPLC was performed on a Varian 5050 (Varian Instrument Co., Palo Alto, CA) chromatograph equipped with a Zorbax ODS (4.6 x 250 mm) column (Dupont Instrument Co., Wilmington, DE) whose eluate was monitored for UV absorbance (UV-100, Varian Instrument Co.) and fluorescence (Ex. 360 nm, Em. 430 nm; MPF-2A, Perkin-Elmer, Norwalk, CT). Metabolites were isolated and UV spectra determined on-line with a Dupont 850 HPLC fitted with a Zorbax 9.4 x 250 mm ODS column and a Perkin-Elmer LC-85B spectrophotometric detector.

RESULTS

Mutagenicity of 3-Nitrofluoranthene and its Reduced Derivatives

The mutagenicity of 3-NFA (Fig. 1) towards *Salmonella typhimurium* strain TA98 (Fig. 2) is predominantly direct-acting (4 200 His⁺ rev/nmole) and is decreased by the presence of S9 to 250 rev/nmole. In strain TA98NR, deficient in the "classical" nitroreductase enzyme (7) and in the variant TA98/1,8-DNP₆ apparently deficient in a transacetylase enzyme (7), activity is lower than in the fully competent TA98 (800 and 475 rev/nmole respectively), and is not restored by addition of S9 (125 rev/nmole in TA98NR; no activity above background in TA98/1,8-DNP₆)

3-AFA, the final product of 6-electron reduction of 3-NFA, is less active in all three variants of TA98 than is 3-NFA (highest values 100 rev/nmole in TA98 and TA98NR in the presence of S9; see Fig. 3). The mutagenicity of 3-AAFA is even lower, 60 rev/nmole or less, and is entirely S9-dependent in all three variants (Fig. 4).

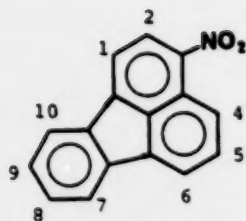


FIGURE 1.
3-Nitrofluoranthene.

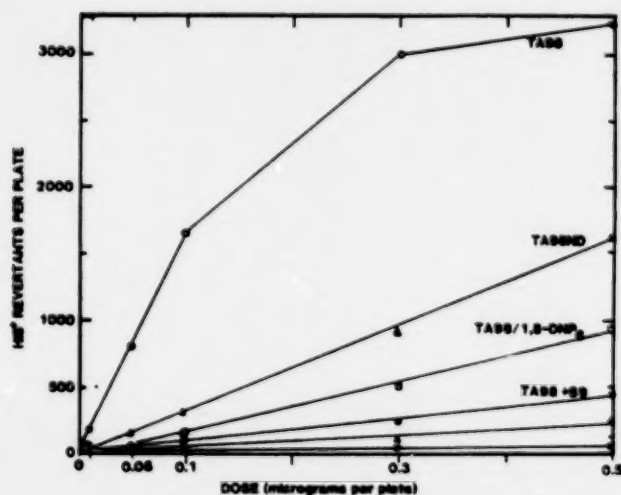


FIGURE 2. Mutagenicity of 3-Nitrofluoranthene towards *Salmonella typhimurium* strains TA98, TA98NR and TA98/1,8-DNP₆ with (closed symbols) and without (open symbols) metabolic activation (S9). Assays were conducted as described under Materials and Methods.

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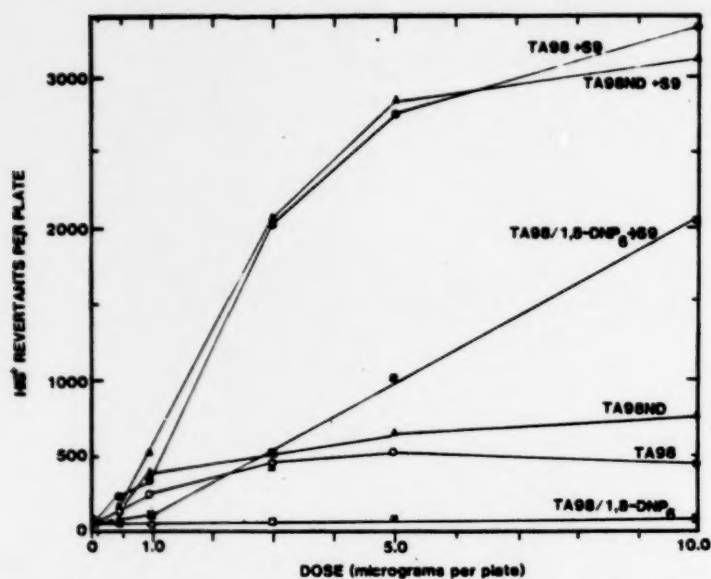


FIGURE 3. Mutagenicity of 3-Aminofluoranthene in the Ames plate incorporation assay (see legend to Figure 2).

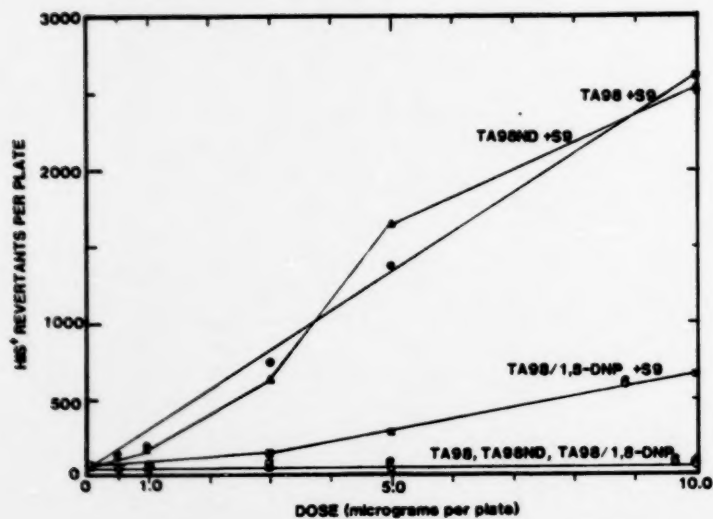


FIGURE 4. Mutagenicity of 3-Acetamidofluoranthene towards *Salmonella typhimurium* in the Ames plate incorporation assay (see legend to Figure 2).

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TABLE 1. Mutagenicity of 3-Nitrofluoranthene toward TM677.

Dose (μg per ml)	Without S9		With S9	
	Mutant Fraction ^a	Percent Survival	Mutant Fraction	Percent Survival
0 (DMSO alone)	1	100	3	100
1.25	1	55	6	200
2.5	1	37	41	115
5	2	43	88	87
10	4	68	94	103
20	1	37	4	47

^a 8-Azaguanine-resistant mutants per 10^5 survivors. Forward Mutation Assay was carried out as described under Methods

In the Forward Mutation Assay 3-NFA was moderately active, and highly toxic, without S9. In the presence of S9 activity was much enhanced and toxicity decreased (Table 1).

Metabolism of 3-NFA by Aroclor-treated Rat Liver S9

After 1 h of incubation at 37°C , over 40 % of the substrate 3-NFA had undergone metabolic transformation (20 nmole/mg protein in 1 h). The ethyl acetate/acetone-extractable metabolites were separated and quantitated by HPLC (Fig. 5). Minor quantities of ^{14}C co-chromatographed with authentic 3-AAFA, and hardly any (< 1%) with 3-AFA. Small amounts of ^{14}C eluting between the solvent front and 3-AAFA were converted to material with a retention time around 17 min (in this HPLC system) by treatment with 1 M HCl for 30 min at 50°C . The major portion of the radioactivity (other than parent compound, which was identified by its retention time and UV-vis spectrum) consisted of a broad peak eluting between 17 and 18 min. This peak was collected and re-chromatographed at 85 % MeOH in H_2O at 2 ml/min on a semi-preparative Zorbax ODS column. Six distinct fractions were detected by UV absorbance and collected separately. All six showed UV-vis spectra which differed from each other but all possessed broad absorbance peaks in the regions 260 - 290 nm and 340 - 400 nm characteristic of NO_2PAH in general and the parent 3-NFA in particular (Fig. 6). All spectra showed peak broadening and shifting to longer wavelengths relative to 3-NFA, and bathochromic shifts on addition of NaOH which were reversed by neutralising with HCl (shown in Fig. 7 for the first (AA) and last (E) fractions in order of elution). HRGC/MS analysis of this fraction indicated a molecular ion of m/e 263, corresponding to the expected molecular weight of 3-NFA-OH (Fig. 8). The loss of 16 mass units ($M^+ - 16$, to

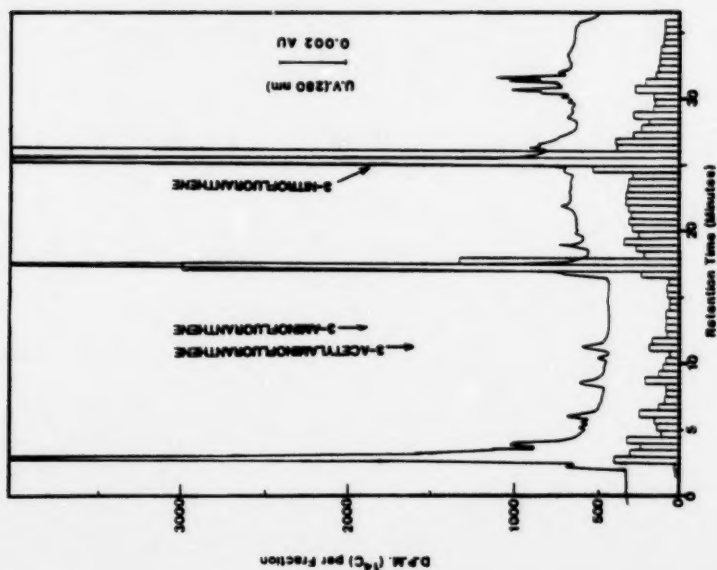


FIGURE 5. HPLC Chromatogram of ethyl acetate/acetone extractable metabolites formed by Aroclor-treated rat liver S9. The solid trace represents UV absorbance at 280 nm, the blocks radioactivity.

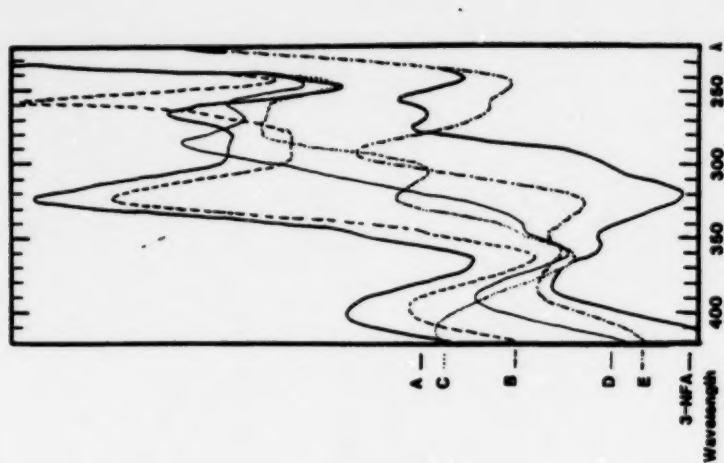


FIGURE 6. UV Spectra of 3-nitrofluoranthrenols A-E and of 3-NFA, in 85% MeOH in H₂O, recorded on-line from an HPLC eluate with a Perkin-Elmer spectrophotometer.

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yield m/e 247) is characteristic of phenols in NCI. The earliest-eluting fraction (AA) gave the least-marked bathochromic shift (only 10 nm), and an overall reduction in absorbance over the region 350 to 500 nm, in contrast to the general pattern represented by the spectra from fraction E (Fig. 7). A similar

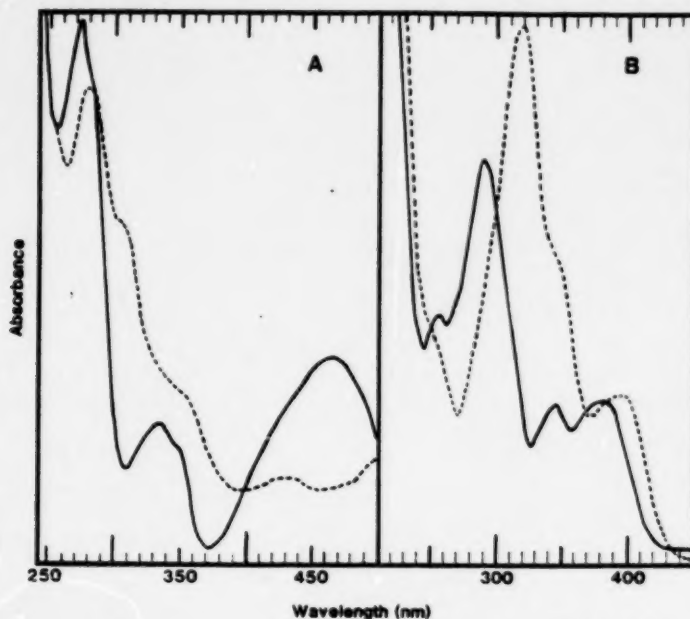


FIGURE 7. UV spectra of 3-nitrofluoranthenols AA (panel A) and E (panel B) in MeOH (solid lines) and in 0.1M NaOH/MeOH (broken lines).

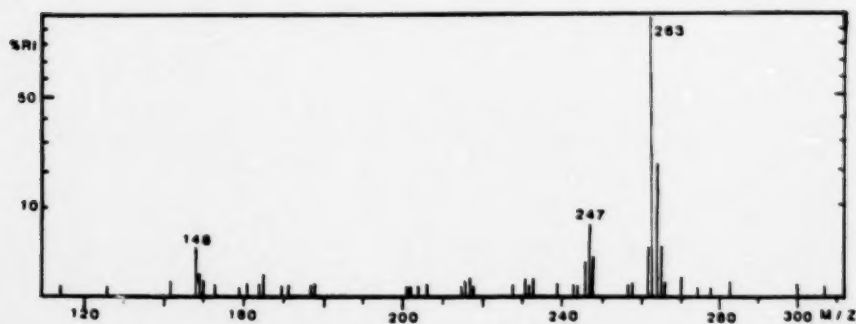


FIGURE 8. NCI mass spectrum of 3-nitrofluoranthenol E

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divergence from the general pattern of the nitropyrenols is exhibited by 1-nitropyren-10-ol (not shown), where the hydroxyl group is peri to the nitro group and the hydroxyl proton can interact with a nitro oxygen to form a stabilised pseudo-six-membered ring. By analogy, it can be inferred that the nitro and hydroxyl groups of AA would be peri to each other, i.e. occupy positions 3 and 4 respectively.

The last phenolic fraction to elute (E) was also the most abundant; its NMR spectrum (Fig. 9) shows clearly the down-field signals due to the protons ortho and peri to the nitro substituent (8.60 ppm, d, H2, and 8.45 ppm, d, H4 respectively) identified by their coupling to H1 (8.16 ppm, d, $J = 7.5$ Hz) and H5 (7.81 ppm, t, $J = 8.2$ Hz, also coupled to H6, 7.98 ppm, d, $J = 6.9$ Hz). The signal at 7.34 ppm is due to an impurity in the solvent acetone- d_6 . The presence of an upfield singlet (7.56 ppm, showing meta coupling 2.5 Hz) and a coupled pair of doublets (7.84, $J = 8.1$, and furthest upfield, 6.96, $J = 8.1$ Hz, further split (2.5 Hz) by meta coupling, indicates that hydroxylation had occurred on the distal benzo ring, at one of positions 8 or 9. Consideration of the strongly directing effects of the nitro group on subsequent electrophilic substitutions (22) suggests that the hydroxyl group would be expected to be found on position 9. Calculation of the effects of the conflicting up-field (due to a hydroxyl group: -0.4 ppm for

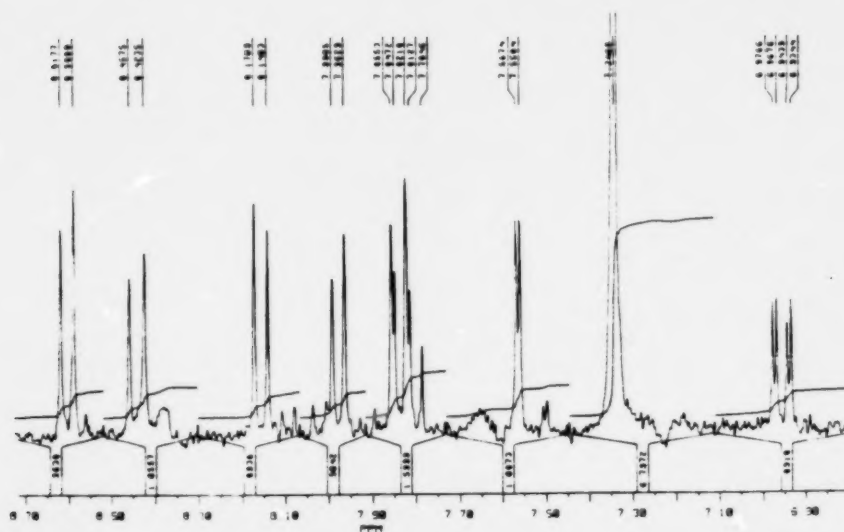


FIGURE 9. 250 MHz Proton NMR spectrum of 3-nitrofluorantheneol E in acetone- d_6 . Signals are in ppm downfield from Me_4Si .

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ortho, meta or para protons [23]) and down-field (from the long-range deshielding effect of the nitro group) shifting influences on the signals due to the protons at positions 7 and 10 (8.06 and 8.12 ppm respectively in 3-NFA; cf 8.01 ppm in the parent fluoranthene) yields chemical shift values more consistent with the occurrence of hydroxylation on position 8, though since the difference in shifts of H7 and H10 (in 3-NFA) is less than 0.1 ppm, and the variation in substituent effect shifts is of the order of 0.1 ppm, this argument is not conclusive in favour of 3-NFA-8-OH.

DISCUSSION

Whereas conversion through 1-aminopyrene to 1-acetamidopyrene vastly increased the S9-dependent mutagenicity of 1-NP (12), the present study shows that in the case of 3-NFA metabolism to 3-AAFA would result in detoxication rather than increase the genotoxicity of this compound. Only a small proportion of the mutagenicity of 3-NFA is mediated by the classical nitroreductase enzyme, as shown by its low activity in strain TA98/1,8-DNP₆ which is proficient in this enzyme. In the Forward Mutation Assay 3-NFA is activated most extensively in the presence of S9, suggesting that S9 may itself generate mutagenic species. 3-NFA appears to be reduced less readily by S9 than is 1-NP (12). S9 is capable of hydroxylating 3-NFA on at least six of the nine possible positions on the fluoranthene rings. Epoxidation is suggested by the presence of small amounts of metabolite which chromatographs as a phenol on treatment with acid, and is therefore tentatively identified as a dihydrodiol. Bioassay-directed fractionation studies of ambient air particulate matter (24) indicate that nitrofluoranthene-like compounds contribute substantially to the mutagenicity of organic extracts of air particles, suggesting that these metabolites of 3-NFA as yet uncharacterised with respect to mutagenic potency may have a role to play in the mammalian and human genotoxicity of NO₂PAH.

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METABOLISM AND MUTAGENICITY OF 3-NITROFLUORANTHENE

KEY WORDS:

Instrumentation: HPLC, NMR, HRGC-MS/NCI

Major Chemical Species: 3-Nitrofluoranthene,
3-Aminofluoranthene, 3-Acetamidofluoranthene, nitro-substituted
polycyclic aromatic hydrocarbons

Metabolism by rat liver S9

Mutagenicity of nitro-substituted polycyclic aromatic
hydrocarbons